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Development of Vaccines to Prevent Wound Infections  
Due to Anaerobic Bacteria

ANNUAL REPORT

Dennis L. Kasper, M.D.

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The Peter Bent Brigham Hospital  
A Division of Brigham and Women's Hospital  
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Summary

BACTEROIDES

Anaerobic bacteria are common causes of infections resulting from wounds and B. fragilis is the most likely of the various anaerobes to be isolated in serious infections. Clearly this organism is also involved in the pathogenesis of experimental intraabdominal infection and abscess formation. Immunization of rats with the capsular polysaccharide of B. fragilis induces protection to subsequent challenge with this organism. We have previously shown that in experimental animals protection to bacteremia is mediated by antibodies to the capsule while protection from intraabdominal abscess formation requires the presence of T cells. During this year we have shown that purified T cells obtained from immunized rats transfers protection while B cells and macrophages do not.

During this year major advances were made in defining the chemical nature of the B. fragilis capsular polysaccharide. It is a complex carbohydrate with 10 known monosaccharide constituents. We developed methods of growing and extracting this antigen which improved our yields by twenty fold. We have shown that the chemical constituents are reproducibly present from lot to lot with only minor variation in quantitative composition.

Another important advance which occurred this year was demonstrating that our findings of immunization and spleen cell dependent transfer of immunity could be verified in another animal species (mouse). Finally, we have demonstrated that B. fragilis may play an important role in pelvic inflammatory disease.

An IND application has been completed and when final approval is obtained from the USAMRDC, a phase I vaccine study will be conducted in human volunteers.

I. Modification of growth media and extraction techniques which enhance capsule production and isolation.

All strains of B. fragilis possess a capsular polysaccharide. Immunochemical purification can be achieved by gentle separation of the polysaccharide from the outer membrane (1), although we recently have developed more efficient methods of purification. Capsules can be demonstrated by several techniques (2). The best method for this has been an indirect immunofluorescence assay that was developed for identification of clinical isolates. Capsular material immunologically similar to that in a reference strain of B. fragilis was identified with specific antibodies. In this assay, all 132 strains of B. fragilis that we have studied were positive for capsular material, whereas all but one of 62 strains from the other species were negative. The fluorescence-positive strain of the other species of Bacteroides was B. thetaiotaomicron. We have utilized this IFA test for accurate and rapid identification of B. fragilis directly in clinical specimens (3).

In attempting to purify the B. fragilis capsule, an observation was made which we reported last year and which bears directly on studies of the immunology of the organism. The amount of capsular antigen produced by B. fragilis appeared to decrease significantly with in vitro passage on blood agar plates (4). This decrease in the quantity of extractable capsule is associated with the emergence of a small transparent colony type variant. The small colony type strain also has a glycogen-like material associated with its outer membrane. This glycogen is found intracellularly and is not associated with the outer membrane on the mucoid colony variant that predominates after passage in animals of the large colony type (LCT). No concomitant alteration in outer membrane proteins or lipopolysaccharides is associated with this colonial transformation. Electron micrographs, with specific staining of the capsule, confirmed the relative loss of capsule with in vitro passage. Passage of these in vitro passed B. fragilis through rats in the abscess model (4) caused an increased amount of capsule and the organisms revert to a predominate of LCT. Thus care must be taken during in vitro studies of B. fragilis to define the antigenic structure and the degree of passage of the strain. During this past year we have made significant changes in the methods we have previously used for growing and isolating the B. fragilis capsule.

B. fragilis strains are grown overnight in a pre-reduced anaerobically sterilized medium containing 0.5% yeast extract, 20 g/l Proteose-peptone, 40 g/l glucose, 5 g/l NaCl, 0.55 g/l cysteine HCl and 10% fetal calf serum. The important ingredients which we have found increases the yield of bacteria between 10-50 fold are fetal calf serum and sufficient glucose. The organisms are grown anaerobically at neutral pH (pH titrator) with N<sub>2</sub> bubbling through the medium. Organisms are collected by centrifugation. Capsule can be extracted and purified from outer membranes as we have previously done (5), or from the aqueous phase of a phenol water extract (68° for 30 min.) of the organisms. In either case, the lipopolysaccharide (LPS) is readily separable from the capsule by chromatography of the aqueous

phase on Sephacryl S-300 in a buffer containing 3% sodium deoxycholate (6,7). Contamination of the capsule with the LPS is readily identifiable because the LPS lacks galacturonic acid and fucose but has rhamnose. Furthermore, the LPS contains several long chain fatty acids including 3-OH fatty acids which are present in the capsule (8). The capsule does not disaggregate in this buffer and elutes in the void volume of the Sephacryl column. The fractions containing the capsular antigen are pooled, concentrated and finally purified on an S-300 column equilibrated in 0.05M Tris buffer pH 7.6. After exhaustive dialysis the purified capsule is lyophilized. The yield of purified capsule prepared by this method is 30-40 mg/liter of broth. This is a minimum of 20 fold increase over our previous methods and has allowed us to proceed with chemical analysis because sufficient quantities of capsule can now be prepared.

## II. Chemical analyses of the B. fragilis capsule.

The principal investigator spent the first 6 months of this academic year in Sweden working on the chemical definition of this capsular material. Chemical analysis of the B. fragilis capsule defines this polysaccharide as a complex carbohydrate. This antigen contains no protein or nucleic acid and has less than 0.5% detectable fatty acids (by GLC). Elemental analysis demonstrates no protein, although there is a significant amount (~4%) of the single amino acid, alanine. Hydrolysis of the polysaccharide into monosaccharides followed by conversion of these to alditol acetates demonstrates several interesting monosaccharides present by gas-liquid chromatography on OV225 and OV17.

### Monosaccharide composition (%) of B. fragilis capsule

	Strain 23745	Strain 9343
D galacturonic acid	15	12
L fucose	18	22
D mannose	2	--
D galactose	15	27
D glucose	9	9
D guinivosamine	--	15
L quinivosamine	18	15
D rhamnosamine	9	--
L fucosamine	6	--
D glucosamine	3	--
? 3 amino,6 deoxyhexose	6	?

Two separate strains have been studied (23745 and 9343). These two were chosen because they seemed to be the two strains we have found with the least degree of immunologic cross-reactivity (although they do cross-react). This was determined in the IFA assay using specific antisera prepared in rabbits to each capsule. Although these strains did fluoresce with the heterologous antiserum above the control non-immune serum, the fluorescence was not as strong as that seen with other strains. Carbodiimide reduction

of these polysaccharides indicated that they both had important galacturonic acid constituents. Furthermore, hydrolysis under more severe conditions (4M HCl) indicates that the alanine is likely to be linked to a 3 amino-6 deoxyhexose. These analyses were all done by the principal investigator while working in Sweden with Dr. Alf Lindberg, Jorgen Lönnegren and Bengt Lindberg.

Methylation analysis of these polysaccharides is exceedingly complex and only limited information is available. Fucose is definitely in a terminal position. The reduced polysaccharide has a new trimethyl hexose not present in the non-reduced polysaccharide indicating that the galacturonic acid may also be terminal.

This array of carbohydrates, some of which are unusual and not often found in bacterial polysaccharides will make the chemical definition of the secondary structure of the repeating unit difficult.

Chemical reduction of the galacturonic acid residue to galactose also indicates that the galacturonic acid residue is immunodominant, because the reduced polysaccharide no longer precipitates with specific anticapsular antiserum when tested by double diffusion in agar.

### III. Analyses of lot to lot variability of the monosaccharide constituents.

Utilizing the new technology for growing and extracting B. fragilis polysaccharides, the variability of monosaccharide composition from lot to lot has been analyzed for both strains 23745 and 9343 capsules. We prepared 2 lots of both 23745 and 9343 capsules in Sweden and have now prepared 1 lot of each since returning to Boston. All monosaccharide constituents were comparable from lot to lot. Variability in quantitative composition was not  $\pm 20\%$  for any monosaccharide. Lot 2 of 23745 polysaccharide (prepared at the National Bacteriologic Laboratory in Stockholm) had in general a greater composition of the amino sugars (quinovosamine, rhamnosamine, glucosamine, and fucosamine) than did lot 1 or 3.

### IV. Safety and toxicity testing of lot 1 of polysaccharide antigens from strains 9343 and 23745.

a. Rabbit pyrogenicity: Five rabbits were injected intravenously, each with 100  $\mu$ g of the B. fragilis polysaccharide preparation. Rectal temperatures were recorded before each injection and hourly for five hours after injection.

b. Mouse toxicity: Each of the 10 mice (20g) were injected intraperitoneally with 50  $\mu$ g of polysaccharide in 0.5 ml phosphate buffered saline. The animals were observed and weighed daily for 12 days. Failure to thrive or gain weight when compared to phosphate buffered saline injected controls would have been considered a toxic manifestation, but this was not seen. Animals continued to grow and thrive well.

c. Guinea pig toxicity: Each of the 5 Hartley strain guinea pigs (350g) were injected with 500  $\mu$ g of the capsular polysaccharide in 5 ml PBS. Significant differences from PBS injected controls would have considered a toxic manifestation, but no differences were seen.

d. Rabbit system toxicity: Each of the 5 New Zealand White rabbits (3kg) were injected intravenously with a total of 500  $\mu$ g of polysaccharide in divided doses over a three week period. Comparison to PBS injected controls were done, observing for weight gain for failure to thrive, but no differences were seen.

#### V. Characterization of the cellular nature of protection in experimental animals.

Last year we reported studies on immunity to B. fragilis in an experimental rat model of intraabdominal sepsis (9-14). It had been shown that active immunization of rats with the capsular polysaccharide of B. fragilis protects these animals against abscess development following intraperitoneal challenge with this species. Passive transfer of hyperimmune globulin from immunized animals to non-immune recipients provided protection against abscess development. On the other hand, adoptive transfer of spleen cells from immunized animals to non-immunized recipients resulted in protection against abscesses following challenge with B. fragilis. These data suggested that a cell mediated immune response was involved in protection against abscess development following immunization with B. fragilis capsular antigen.

In order to determine the possible role of cell mediated immunity prompted by the capsular antigen, inbred congenitally athymic OLA/Rnu rats and their phenotypically normal littermates were actively immunized. Despite the development of high titers of anti-B. fragilis capsular antibody, 100% of actively immunized athymic rats developed abscesses, as did 100% of unimmunized athymic control rats. However, no phenotypically normal littermate control rats that were actively immunized developed abscesses, while 100% of phenotypically normal unimmunized rats developed abscesses.

In order to substantiate that this immunity was definitively T cell transferred, adoptive transfer experiments were performed with purified T cells.

Separation of T and B cells from the rat spleen was performed using nylon wool columns. It is known that B cells and macrophages generally adhere to nylon wool, while T and null cells pass through. The methods used were those published by Julius et al. (15). Thirty-five cc syringe columns were set up with 3-way stopcocks and 18 gauge needles. Fifty cc of balanced salt solution (BSS) with 5% fetal calf serum (FCS), which was used as the media throughout, was run through the column. Spleens were removed from immunized rats and cells released by disruption with a stainless steel wire mesh gauze. The cells were passed through a dacron gauze filter to remove debris and counted using a Coulter ZBI counter.  $3 \times 10^8$  spleen cells were



suspended in media to give a volume of 6 cc. Cells were loaded onto the column and incubated at 37°C for 1 hour. Columns were eluted with 50 cc of media at 37°C with an elution rate of 1 drop per second. Cells were spun at 1000 rpm for 15 minutes, brought up in 6 cc BSS, like fractions pooled and counted in the Coulter counter. Fifty cc of BSS at 4°C was run through the columns, again at the rate of 1 drop per second. These fractions were also centrifuged, pooled and counted. Appropriate dilutions were made for cell transfer. This method generally gave a yield of  $1.1 \times 10^7$  cells/ml (22%) with warm (37°C) elution. This is consistent with previously published data for cell yields using nylon wool columns (10). Cold elution (4°C), which elutes B cells and macrophages adherent during warm elution, yielded  $1.6 \times 10^6$  cells/ml. Transfer of these T cell enriched and B cell and macrophage populations to non-immune recipients (see table page 7) indicate that significant protection against abscess development occurs when immune T cell enriched populations are transferred. B cell and macrophage enriched populations from the same spleens failed to transfer immunity to non-immune recipients challenged with *B. fragilis*. The 50% protective dose (PD<sub>50</sub>) for T cell enriched transfer recipients was  $3.2 \times 10^5$  cells, while the PD<sub>50</sub> for the B cell and macrophage recipients was greater than  $2 \times 10^6$  cells. A Chi square comparison of the T cell recipient and B cell and macrophage recipient groups for protection against abscess regardless of the number of cells transferred was significant with a  $P < 0.01$ .

#### VI. Adoption of the model to the mouse.

In an effort to confirm our earlier findings in another host and to facilitate immunogenetic studies, a mouse model for intraabdominal abscess has been developed. Unlike the rat model, the mouse model does not require the use of barium sulfate for abscess production. In addition the inoculum concentration of *B. fragilis* required to produce abscesses is  $1 \times 10^5$  cfu/ml in mice as opposed to  $5 \times 10^7$  cfu/ml in rats, indicating that mice are more sensitive to the inoculum challenge. Immunization of C57 BL/10 mice with capsular polysaccharide yielded results indicating a clear dose response regarding antibody development. No antibody was detected in animals receiving 2.5 mcg/animal or less of capsular antigens and maximum antibody response was noted with a dose of 10 mcg/animal. Challenge of immunized mice with *B. fragilis* at various concentrations yielded the following data:

<u>Inoculum Group</u>	<u>Immune Status</u>	<u>Abscess</u>
<i>B. fragilis</i> $1 \times 10^8$ cfu/ml	None	7/7
<i>B. fragilis</i> $1 \times 10^8$ cfu/ml	10 mcg <i>B. fragilis</i> CP	9/10
<i>B. fragilis</i> $1 \times 10^6$ cfu/ml	None	9/9
<i>B. fragilis</i> $1 \times 10^6$ cfu/ml	10 mcg <i>B. fragilis</i> CP	2/5
<i>B. fragilis</i> $1 \times 10^7$ cfu/ml	Passive $5 \times 10^7$ spleen cells from immune	6/9

At the lower inoculum level of  $1 \times 10^6$  cfu/ml only 2 of 5 immunized animals developed abscess as opposed to all 7 non-immune animals.. Interestingly, even at  $1 \times 10^8$  cfu/ml recipients of spleen cells from immunized animals

Efficacy of Passive Transfer of T Cell Enriched  
or B Cell and Macrophage Populations from  
Immunized Wistar/Lewis Rats Against Challenge with B. fragilis

<u>Immunogen</u>	<u>Cell Density</u>	<u>Abscess Incidence<sup>+,‡</sup></u>
Spleen cells from unimmunized rats	$2.5 \times 10^6$	10/10
Spleen cells from rats immunized with <u>B. fragilis</u> capsular polysaccharide	$2.5 \times 10^6$	0/9
	$8 \times 10^5$	1/5
	$4 \times 10^5$	2/5
	$2.5 \times 10^5$	3/3
T cell enriched spleen cells from rats immunized with <u>B. fragilis</u> capsular polysaccharide	$2.5 \times 10^6$	1/10
	$8.0 \times 10^5$	1/5
	$4.0 \times 10^5$	2/5
	$2.5 \times 10^5$	3/4
B cell and macrophage enriched spleen cells from rats immunized with <u>B. fragilis</u> capsular poly- saccharide	$2 \times 10^6$	3/5
	$8 \times 10^5$	4/5
	$4 \times 10^5$	3/4

\* Viable cell density transferred to non-immune recipients

<sup>+</sup> Comparison of total group R to group S Chi square analysis (P<0.01)

<sup>‡</sup> Protective dose for 50% of animals calculated by Reed-Muench method.  
Group Q PD<sub>50</sub> =  $3.5 \times 10^5$  cells, Group R PD<sub>50</sub> =  $3.2 \times 10^5$  cells and  
Group S PD<sub>50</sub> greater than  $2 \times 10^6$  cells

show some reduction in abscess development. These preliminary data indicate: 1) the abscess model produced with rats can be adapted for C57 BL10 mice; 2) mice immunized with B. fragilis capsular polysaccharide have reduced incidence of abscess development when challenged with B. fragilis; and 3) immunity to abscess development can be passively transferred to non-immune animals with spleen cells from immunized mice.

VII. Serological evidence for the role of B. fragilis and enterobacteriaceae in the pathogenesis of acute pelvic inflammatory disease.

The incidence of acute pelvic inflammatory disease (PID, acute salpingitis) - one of the most common gynaecological disorders - seems to be rising (16). It mostly affects young women, and its late sequelae are often severe: involuntary sterility, increased risk of ectopic pregnancies, and chronic abdominal pains (17).

Except for the role of the sexually transmitted organisms, Neisseria gonorrhoeae and Chlamydia trachomatis, the pathogenesis of PID is poorly understood. Modern techniques of anaerobic bacteriology of pus samples obtained by culdocentesis suggest that obligate anaerobic bacteria may often be involved (18). However, a variety of facultative bacteria are also seen in such cultures, and the interpretation of the culture results is confused by frequent contamination of the samples with the abundant and complex cervicovaginal flora.

The determination of antibodies to the capsular polysaccharide of Bacteroides fragilis with radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) has been found useful in the serological diagnosis of B. fragilis infections (19,20). Enterobacterial common antigen (21) (ECA) is common to all members of the large family of enteric bacteria, and anti-ECA determination can be used to detect their presence (22).

#### Patients and Methods

##### Patients

The study population consisted of 101 consecutive women with acute PID not associated with surgery or instrumentation who, because of severe symptoms, were admitted to the University Central Hospital, Helsinki, between January, 1978, and August, 1979. The clinical diagnosis of acute PID was based on common criteria (23) which included lower abdominal pain, vaginal discharge, adnexal tenderness with or without pelvic mass, erythrocyte-sedimentation rate  $\geq 15$  mm/h, and usually fever ( $\geq 38^{\circ}\text{C}$ ).

The mean age of the 101 patients was 27.9 years (range 15-47), and 50 were aged under 30. 56 patients were nulliparous, 50 were fitted with an intrauterine device (IUD), and 11 were taking oral contraceptives. Mean duration of symptoms before examination was 8.2 days. Gynaecological examination revealed acute PID without palpable adnexal mass in 60 patients, and PID with such a mass on one or both sides in 41 patients.

### Serological and Microbiological Methods

Cervical and urethral specimens for culture of *N. gonorrhoeae* and *C. trachomatis* were collected at the time of admission and handled by conventional methods as described elsewhere (24). Irradiated McCoy cells were used for isolation of chlamydia (25). Both acute-phase and convalescent-phase sera (interval 1 to 3 weeks) were collected from all patients and stored at -20°C until both sera of each pair could be assayed together on the same day.

#### Serological Findings in Paired Sera of 101 PID Patients

Serological Findings	Anti- Chlamydia (IFT)	Anti-ECA (hema- glutination)	Anti- <i>B. fragilis</i> (ELISA)	Any of the three tests
Positive:*	32	30	28	64
The only positive finding	17	14	12	43
Together with one other	10	11	11	16
All three positive	5	5	5	5
Change in titre only <sup>†</sup>	6	15	18	--
High value only <sup>‡</sup>	14	9	7	--
High value <sup>‡</sup> plus change <sup>‡</sup>	12	6	3	--

\* One or several of the criteria listed below. <sup>†</sup> <sup>‡</sup>

<sup>†</sup>  $\geq 4$  fold change in titre of the anti-ECA or anti-Chlamydia assays or  $\geq 1.6$  fold change in OD in the anti-*B. fragilis* assay.

<sup>‡</sup> Reciprocal titre for anti-Chlamydia  $\geq 512$ , for anti-ECA  $\geq 4096$ , or for anti-*B. fragilis* an OD  $\geq 2.5$  (all values seen in less than 2% of healthy adults in Finland).

Antibodies to ECA were determined by passive hemagglutination using human group O red blood cells coated with ECA<sup>+</sup> and ECA<sup>-</sup> (control) extracts of *Salmonella typhimurium* (22). IgM antibodies to the capsular polysaccharide of *B. fragilis* were assayed with an ELISA test (V.V. Valtonen, A. Palmu, B.J. Mansheim, D.L. Kasper, unpublished); and the results are given as corrected optical density (OD) values. IgG antibodies to *C. trachomatis* were determined with an immunofluorescence test (IFT), in which inclusions of *C. trachomatis* serotype L2(434 Bu) in cell culture served as antigen (26). The criteria for positive findings in these assays were either a significant change in antibody concentration or a value above the 99th (for anti-ECA the 98th) percentile determined from series of sera from apparently healthy Finnish adults (29) (M. Malkamäki, unpublished; V.V. Valtonen, unpublished) (table above).

Significance of the findings was tested with the  $\chi^2$  test or the F test as appropriate.

### Results

Positive serological findings in any of the three tests (anti-ECA, anti-*B. fragilis* ELISA, anti-*Chlamydia* IFT) were observed in 64 patients (table above). Sixteen of them had positive results in all three tests. Eleven patients were concomitantly seropositive for ECA and *B. fragilis*. Of the 30 positive tests for anti-ECA, 21 (70%) comprised a change in titre (all except one were rises) and 9 (30%) a high titre only. Of the 23 positive tests for anti-*B. fragilis*, 21 (75%) comprised a rise in the OD value and 7 (25%) a high value only. Of the anti-*C. trachomatis* findings, 18 (56%) were titre changes (14 rises, 4 falls) and 14 (44%) were high titres.

Cultures from the lower genital tract were positive for *N. gonorrhoeae* in 25 patients and for *C. trachomatis* in 32 patients. Positive serological findings for ECA or *B. fragilis* were evenly distributed (in 25 to 32% of the cases) in the groups with positive and negative culture results for either of these agents (see table below). By contrast, positive serology

CORRELATION OF SEROLOGY WITH CULTURES FROM THE LOWER GENITAL TRACT

Lower genital tract cultures	Seroology positive for				Seroology negative
	No.	<i>C. trachomatis</i>	ECA	<i>B. fragilis</i>	
<i>N. gonorrhoeae</i> +	25*	9 (36%)	10 (40%)	7 (28%)	3 (12%)
<i>N. gonorrhoeae</i> -	75	23 (31%)	23 (31%)	21 (28%)	28 (37%)
<i>C. trachomatis</i> +	32†	18 (56%)	15 (47%)	17 (53%)	5 (16%)
<i>C. trachomatis</i> -	69	14 (20%)	22 (32%)	13 (19%)	32 (46%)

\*As defined in the footnotes to table I.

†Includes 6 patients positive for both *N. gonorrhoeae* and *C. trachomatis*.

‡Difference significant;  $P < 0.01$  (2x2 test).

for *C. trachomatis* correlated with the isolation of the agent from the lower genital tract, although it did not correlate with the isolation of *N. gonorrhoeae*.

Adnexal mass was present in 9 of 37 (24%) patients who had negative serology, in 18 of 43 (42%) patients who had one positive serological test, in 9 of 16 (56%) patients who had two positive tests, and in all 5 (100%) patients who had all three tests positive (see table below). In total,

CORRELATION OF SEROLOGY WITH SEVERITY OF DISEASE

Cumulative no. of positive serological findings*	Adnexal mass present (no./total no.)
0	9/37 (24%)
1	18/43 (42%)
2	9/16 (56%)
3	5/5 (100%)

\*Sera tested for antibodies to *C. trachomatis*, ECA, and *B. fragilis*; positive findings defined as in the footnotes to table I.

positive serology was more common in patients with adnexal mass than in those without (see table below). The difference was significant ( $p < 0.05$ )

SEROLOGICAL FINDINGS IN PID PATIENTS WITH OR WITHOUT PALPABLE ADNEXAL MASS

Adnexal mass	No.	Serology positive* for			Serology negative
		<i>C. trachomatis</i>	ECA	<i>B. fragilis</i>	
+	41	19 (46%)†	17 (42%)‡	15 (37%)	9 (22%)§
-	60	13 (22%)†	13 (22%)‡	13 (22%)	28 (47%)§

\*Positive findings defined as in the footnotes to table 1.

†Difference significant,  $\chi^2 = 5.75$ ,  $p < 0.05$ .

‡Difference significant,  $\chi^2 = 4.57$ ,  $p < 0.05$ .

§Difference significant,  $\chi^2 = 5.18$ ,  $p < 0.05$ .

for anti-ECA and anti-*C. trachomatis*, whereas the trend for anti-*B. fragilis* was similar but slightly less pronounced. On the other hand, the presence of an adnexal mass did not correlate with the culture results from the lower genital tract. Culture for *N. gonorrhoeae* was positive in 11 of 41 (27%) patients with adnexal mass and in 15 of 60 (25%) without. The corresponding figures for *C. trachomatis* were 11 of 41 (27%) and 21 of 60 (35%).

Positive serology correlated with a longer mean duration of symptoms (10.6 days compared with 4.5 days,  $p < 0.001$ ) and with IUD use (39 of 64 patients compared with 11 of 37,  $p < 0.01$ ). There was no correlation with a longer mean stay in hospital or with previous PID (see table below).

CHARACTERISTICS ASSOCIATED WITH POSITIVE AND NEGATIVE SEROLOGY

	Serology*		
	All patients	Positive	Negative
No. of patients	101	51	50
Mean age (yr)	27.9	27.2	28.7
Mean duration (days) of symptoms before admission	8.2	10.6	4.5
Mean duration (days) of hospitalisation	8.9	9.4	8.2
Recurrent disease	20	12	8
Adnexal mass	41	32	9
IUD†	50	39	11

NS = not significant.

\*Sera tested for antibodies to *C. trachomatis*, ECA, and *B. fragilis*; positive findings as defined in the footnotes to table 1. Positive serology = a positive finding in any one assay; negative serology = positive findings in none of the assays.

†Intrauterine contraceptive device.

‡F test.

§ $\chi^2$  test.

Whereas adnexal mass was somewhat more frequently found in the patients with IUD (50%) than those without (31%), this difference was not statistically significant.

### Discussion

The two methods that have traditionally been used to investigate the microbial etiology of acute PID are cultures of either endocervical (lower-genital-tract) swabs or pus from the pelvic abscesses. In the endocervical cultures, only *N. gonorrhoeae* and *C. trachomatis* can be considered of etiological significance. But cultures from pelvic abscesses very often yield mixed cultures of aerobic and anaerobic bacteria, and their value is difficult to interpret because of possible contamination by the complex normal vaginal flora. As a different approach, we collected acute-phase and convalescent sera from 101 PID patients and carried out antibody tests for enteric bacteria (anti-ECA test, detecting antibodies to a component common to all members of the Enterobacteriaceae family), *B. fragilis*, and *C. trachomatis*, in the belief that a serum antibody response would be a more clear-cut indicator of the involvement of a bacterial species in the pelvic infection.

We could indeed show positive serological findings in one or more of these tests in 64% of the patients. For anti-ECA and anti-*B. fragilis*, the positive test results were titre changes in 74% of the cases, and all except one of these were increases in titre. Similar titre changes are infrequent in paired sera from healthy persons. For *C. trachomatis*, a larger proportion (44%) of the positive findings were high titres without a significant change. This seems consistent with the view that *C. trachomatis* infections are often more or less chronic, and thus the timing of the serum samples may not have been ideal. It is also possible that in some cases the relatively high levels of antibody were remains from a previous infection. However, this was probably not very often the case since positive serology did not correlate with previous history of PID.

Thirty-seven of the 101 patients in our PID series remained seronegative for enteric bacteria, *B. fragilis* and *C. trachomatis*. Besides these bacteria and *N. gonorrhoeae* (for which a sensitive antibody assay is still lacking) culture studies from pus obtained by culdocentesis have often found anaerobic gram-positive cocci. A serological test to detect an immune response to these and to *Mycoplasma hominis* might have further reduced the seronegative fraction.

A larger proportion of the patients had positive serology either for enteric bacteria or for *B. fragilis*, and a smaller proportion (11 out of 58) were simultaneously positive for both. This suggests an interesting difference in the microbial etiology of upper-genital-tract infection compared with intra-abdominal sepsis originating in the gastrointestinal tract. In the latter, positive serology both for enteric bacteria and for *B. fragilis* very often occurs simultaneously. However, the patients who had more severe PID characterized by palpable adnexal mass showed more often positive serology than those who had PID without such a mass. Furthermore, there was a clear-cut correlation between severity of the disease and the cumulative rate of seropositivity in the three tests applied.

Unexpectedly, we found that the isolation of N. gonorrhoeae and/or C. trachomatis from the lower genital tract did not identify those patients who were prone to have serological evidence of enterobacterial or B. fragilis infection, or who developed an inflammatory adnexal mass. By contrast, earlier studies have suggested that B. fragilis would have more importance in acute non-gonococcal than gonococcal PID (18,19) and that the former would more often lead to abscess formation (23). These differences most likely reflect differences in the patient populations (28).

The present findings do not support the clinical usefulness of differentiating PID patients into gonococcal and non-gonococcal or chlamydial and non-chlamydial PID. The PID patients with and without cervical gonorrhoea in this study were equally likely to have serological evidence of the involvement of enteric bacteria or B. fragilis in their upper-genital-tract infection. The value of lower-genital-tract cultures in PID should thus be recognized as primarily preventive, to motivate the sex partners to seek treatment. From the individual patient's point of view, lower-genital-tract cultures for N. gonorrhoeae and C. trachomatis would be more important in the women who attend the clinics for reasons other than PID, many of whom would otherwise go untreated with increased risk of developing PID.

In our series, the overall frequency of IUD use was as high (50%). Only 11% of the patients used oral contraception. This accords well with earlier studies (29-32). The present increased use of IUD coincides with the increased prevalence of acute PID. Several more or less well controlled studies have also indicated a causal relationship, so that women using an IUD have a risk of acute PID 2 to 9 times higher than other women (29-35). In the present series, significantly more IUD users than non-users had positive serology. This further supports an in vitro study (36) which shows that the tails of an IUD enhance the passage of vaginal and cervical flora to the upper genital tract. Although IUD use may sometimes have led to increased antibacterial antibody levels without overt infection, it is perhaps more likely that the symptoms and signs interpreted as mild side-effects of IUD use may actually indicate subclinical PID, in which potentially pathogenic cervicovaginal bacteria and Chlamydia may be involved. Prospective serological studies in IUD users might help to determine such relationships.

The present study has provided new serological evidence for an important role of aerobic enteric bacteria and encapsulated B. fragilis in acute PID in addition to the known pathogens N. gonorrhoeae and C. trachomatis. These findings also give further support to a polymicrobial etiology for the disease; and this should be remembered in its treatment.



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